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PRODUCTION OF 3-HYDROXYPROPIONIC ACID IN RECOMBINANT ORGANISMS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Patent Application S.N. 60/151,440 filed August 30, 1999.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The research project which gave rise to the invention described in this patent application was supported by EPA grant R824726-01. The United States Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

The technology of genetic engineering allows the transfer of genetic traits between species and permits, in particular, the transfer of enzymes from one species to others. These techniques have first reached commercialization in connection with high-value added products such as pharmaceuticals. The techniques of genetic engineering are equally applicable and cost effective when applied to genes and enzymes which can be used to make basic chemical feedstocks.

A metabolic pathway of interest exists in the bacteria *Klebsiella pneumoniae*, which has the ability to biologically produce 3 - hydroxypropionaldehyde from glycerol.

20 Native microorganisms have the ability to produce 1,3 - propanediol from glycerol as well. Commercial interests are exploring the production of 1,3 - propanediol from glycerol or glucose, in recombinant organisms which have been engineered to express the enzymes necessary for 1,3 - propanediol production from other organisms.

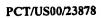
3 - hydroxypropionic acid CAS registry Number [503-66-2] (abbreviated as 3-25 HP) is a three carbon non-chiral organic molecule. The IUPAC nomenclature name for

this molecule is propionic acid 3 - hydroxy. It is also known as 3 - hydroxypropionate, B - hydroxpropionic acid, β - hydroxypropionate, 3 - hydroxypropionic acid, 3 hydroxypropanoate, hydracrylic acid, ethylene lactic acid, β -lactic acid and 2 deoxyglyceric acid. Applications of 3-HP include the manufacture of absorbable 5 prosthetic devices and surgical sutures, incorporation into beta-lactams, production of acrylic acid, formation of trifluromethylated alcohols or diols, polyhydroxyalkonates, and co-polymers with lactic acid. 3-HP for commercial use is now commonly produced by organic chemical syntheses. The 3-HP produced and sold by these methods is relatively expensive, and it would be cost prohibitive to use it for the production of 10 monomers for polymer production. As discussed below, some organisms are known to produce 3-HP. However, there is not yet available a catalog of genes from these organisms and thus the ability to synthesize 3-HP using the enzymes natively responsible for the synthesis of that molecule in the native hosts which produce it does not now exist.

In addition to its commercial utility, 3-HP it is found in a number of biological processes, notably including many naturally occurring bio-polymers. Poly(3 hydroxybutyrate) (PHB) is the most abundant member of the microbial polyesters which contain hydroxy monomers termed polyhydroxyalkonates (PHAs). PHB has utility as a biodegradable thermoplastic material and the material was first produced industrially in 20 1982.

The majority of published research on PHA's that contain 3-HP has concentrated on two bacterial sources: Ralstonia eutropha ("Alcaligenes eutrophus") and Pseudomonas oleovorans. Both Ralstonia eutropha and Pseudomonas oleovorans are able to grow on a nitrogen free media containing 3 - hydroxy - propionic acid, 1,5 -25 pentanediol or 1.7 - heptanediol. When 3-HP is the major hydroxy-acid added to the growth media, poly(3 - hydroxybutyrate - co - 3 - hydroxypropionic acid) is formed containing 7 mol % 3 - hydroxypropionic acid. These cells also store 3 mol %, 3 hydroxypropionic acid poly(3 - butyrate - co - 3 - hydroxypropionic acid).

Recombinant systems have been used to create PHAs. An E. coli strain 30 engineered to express PHA synthase from either Ralstonia eutropha or Zoolgoea ramigera produced poly(3 - hydroxypropionic acid) when feed 1,3 - propanediol.



Skraly, F. A. "Polyhydroxyalkanoates Produced by Recombinant E. coli." Poster at Engineering Foundation Conference: Metabolic Engineering II, 1998. An E. coli strain that expressed PHA synthase (MBX820), when provided with the genes encoding glycerol dehydratase and 1,3 - propanediol dehydratase from K. pneumonia, and 4 - hydroxybutyral- CoA transferase from Clostridium kluyveri, synthesized PHB from glucose.

Glycerol dehydratase, found in the bacterial pathway for the conversion of glycerol to 1,3 - propanediol, catalyzes the conversion of glycerol to 3 - hydroxypropionaldehyde and water. This enzyme has been found in a number of bacteria including strains of Citrobacter, Klebsiella, Lactobacillus, Entrobacter and Clostridium. In the 1,3 - propanediol pathway a second enzyme 1,3 - propanediol oxido-reductase (EC 1.1.202) reduces 3 - hydroxypropanaldehyde to 1,3 - propanediol in a NADH dependant reaction. The pathway for the conversion of glycerol to 1,3 - propanediol has been expressed in E. coli. Tong et al., Applied and Environmental Microbiology 57 (12) 3541-3546. The genes responsible for the production of 1,3 - propanediol were cloned from the dha regulon of Klebsiella pneumoniae. Glycerol is transported into the cell by the glycerol facilitator, and then converted into 3 - hydroxy - propionaldehyde by a coenzyme B₁₂- dependent dehydratase. E. coli lacks a native dha regulon, consequently E. coli cannot grow anaerobically on glycerol without an exogenous electron acceptor such as nitrate or fumarate.

Aldehyde dehydrogenases are enzymes that catalyze the oxidation of aldehydes to carboxylic acids. The genes encoding non-specific aldehyde dehydrogenases have been identified in a wide variety of organisms e.g.; ALDH2 from Homo sapiens, ALD4 from Saccharomyces cerevisiae, and from E. coli both aldA and aldB, to name a few. These enzymes are classified by co-factor usage, most require either AND+, or NADP+ and some will use either co-factor. The genes singled out for mention here are able to act on a number of different aldehydes and it likely that they may be able to oxidize 3 - hydroxy - propionaldehyde to 3 - hydroxypropionic acid.



The present invention is intended to permit the creation of a recombinant microbial host which is capable of synthesizing 3-HP from a starting material of glycerol or glucose. The glycerol or glucose is converted to 3 -

5 hydroxypropionicaldehyde (abbreviated as 3-HPA) which is then converted to 3-HP.

This process requires the so-called *dhaB* gene from *Klebsiella pneumoniae* which encodes the enzyme glycerol dehydratase any one of four different aldehyde dehydrogenase genes to convert 3-HPA to 3-HP. The four aldehyde dehydrogenase genes used were *aldA* from the bacterium *E. coli*, *ALDH2* from humans, *ALD4* from the yeast *Saccharomyces cerevisiae*, and *aldB* from *E. coli*. The yeast gene appeared to give the best results.

It is an object of the present invention to provide a genetic construct which encodes glycerol dehydratase and aldehyde dehydrogenase enzymes necessary for the production of 3 - hydroxypropionic acid from glycerol.

It is also an object of the present invention to provide a method for the production of 3 - hydroxypropionic acid from glycerol.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiment thereof and from the claims.

20 BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS
Not applicable.

DETAILED DESCRIPTION OF THE INVENTION

It is disclosed here that it is possible to introduce into a bacterial host genes encoding two enzymes and thus confer upon that host the ability to produce 3-HP from glycerol. The two necessary enzymes are glycerol dehydratase and aldehyde dehydrogenase. It is here reported that the two enzymes are both necessary and sufficient to enable a strain of a suitable host, such as a competent *E. coli* strain, to make 3-HP from glycerol. An exemplary gene encoding a glycerol dehydratase is known, the dhaB gene from Klebsiella pneumoniae, sequenced and rendered convenient to use.

30 Several exemplary aldehyde dehydrogenases are known, and their sequences are

presented here. From this information, it becomes practical to confer upon a bacterial host the ability to convert glycerol into 3-HP in a commercially reasonable manner.

It was not apparent before the completion of the work described here that these two diverse enzymes could be produced in a common host to produce the ability to 5 make 3-HP. There are many known aldehyde dehydrogenase enzymes and genes, and the enzymes are known to have varying substrate specificities and efficiencies. There was not evidence, prior to the work described here, that the aldehyde dehydrogenase enzyme would work on the 3-hydroxypropionicaldehyde (3-HPA) substrate to create 3-HP. Without that knowledge, there was no data from which to predict the effectiveness 10 of the 3-HP production studies described below. An additional uncertainty arises from the fact that the intermediate aldehyde, 3-HPA, is toxic to many bacterial host and thus the survival of the host is dependent upon the relative rates of enzymatic production and conversion of the aldehyde intermediate to non-toxic 3-HP.

A difficulty in the realization of the production of 3-HP desired here is that 15 ribosome binding sites from non-native hosts are often ineffectual and lead to poor protein production and that many non-native promoters are often poorly transcribed and a bar to high protein expression. However, the inventors also recognized that a nonnative promoter that is known to be very active and is inducible by the addition of a small molecule unrelated to the pathway being expressed is often a very efficient way to 20 express and regulate the levels of enzymes expressed in hosts such as E. coli. To achieve high levels of regulated gene expression plasmids were constructed which placed the expression of all exogenous genes necessary for the production of 3 hydroxypropionic acid from glycerol under the regulation of the trc promoter. The trc promoter, is efficient, not native to E. coli, and inducible by the addition of IPTG.

The present specification describes a genetic construct for use in the production of 3 - hydroxypropionic acid from glycerol. The genetic construct includes exemplary DNA sequences coding for the expression of a glycerol dehydratase and a DNA sequence coding for aldehyde dehydrogenase. The set of exemplary sequences necessary for the expression of glycerol dehydratase is collectively referred to as 30 "dhaB". The set of sequences necessary for the expression of aldehyde dehydrogenase includes any one of four different genes which proved efficacious. The individual

aldehyde dehydrogenase sequences referred to individually as ALDH4, ALD2, aldA and aldB.

Producing 3 - hydroxypropionic acid in a foreign host

In the work described below, the enzymes necessary for the production of 3
5 hydroxypropionic acid from glycerol in *E. coli* were expressed under the regulation of the *trc* promoter, a non-native promoter inducible by the addition of IPTG. The glycerol dehydratase was encoded by the *dhaB* gene from *Klebsiella pneumoniae*, the aldehyde dehydrogenases used was any one of four different genes (*ALDH2* from *Homo sapiens*, *ALD4* from *S. cerevisiae*, *aldB* from *E. coli* or *aldA* from *E. coli*). Expression of these genes coding for glycerol dehydratase and any one of the genes encoding an aldehyde dehydrogenases was sufficient to enable the construct to produce 3-HP when the fermentation media was supplemented with glycerol. In all of these constructs, the *dhaB* gene was downstream from the gene encoding the aldehyde dehydrogenase used, and expression of both genes was regulated by the *trc* promoter. This order, however, is not required and the order of the gens on a construct and the use of multiple constructs is possible.

In a minimal genetic construct made based on the data presented here, the only genetic elements present that would be necessary are the structural genes *dhaB* and an aldehyde dehydrogenase gene encoding a protein that efficiently catalyzes the oxidation of 3-hydroxypropionaldehyde to 3-hydroxypropionic acid, and non-native promoter sequences specifically selected to give the type of inducible control most appropriate for the context of the process in which the construct is to be used. Extraneous pieces of DNA, whether retained in the construct or added from other DNA sequences, would not necessarily be detrimental to effective 3-HP synthesis by the host organism, but would not be needed. Each sequence to be translated would necessarily be preceded by a ribosome binding site, functional in the selected host so that the messenger RNA(s) coding for the proteins of interest could be translated by ribosomes. Terminator sequences immediately downstream of each translated unit would also be necessary in some organisms, particularly in eukaryotes. The construct could be part of an

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integrated into the genome of the host.

The structural genes and appropriate promoter(s) could be isolated by the use of restriction enzymes, by the polymerase chain reaction (PCR), by chemical synthesis of the appropriate oligonucleotides, or by other methods apparent to those skilled in the art 5 or molecular biology. The promoter(s) would be derived from genomic DNA of other organism or from artificial genetic constructs containing promoters. Appropriate promoter fragments would be ligated into the construct upstream of the structural genes in any one of several possible arrangements.

The aldehyde dehydrogenase expressed would have: high specific activity 10 towards 3-hydroxypropionaldehyde; be very stable in the host it is expressed in; be readily over expressed in the selected host; not be inhibited by either the substrates necessary for the reaction or the products formed by the reaction; be fully active under the fermentation conditions most favorable for the production of 3 - hydroxypropionic acid and be able to use either NAD+ or NADP+.

One possible arrangement is the true operon, where one promoter is used to direct transcription in one direction of all necessary Open Reading Frames (ORFs). The entire message is then contained in one messenger RNA. The advantages of the operon are that it is relatively easy to construct, since only one promoter is needed; that is it is relatively simple to replace the promoter with another promoter if that would be 20 desirable later; and that it assures that the two genes are under the same regulation. The main disadvantage of the operon scheme is that the levels of the expression of the two genes cannot be varied independently. If it is found that the genes, for optimal 3 hydroxypropionic acid synthesis, should be expressed at different levels, the operon in most cases cannot be used to realize this.

Another possible arrangement is the multiple-promoter scheme. Two or more promoters, with the same or distinct regulatory behavior, could be used to direct transcription of the genes. For example, one promoter could be used to direct transcription of dhaB and one to direct transcription of the gene encoding the appropriate aldehyde dehydrogenases. Because the genes theoretically can be 30 transcribed and translated separately, a great number of combinations of multiple promoters is possible. Additionally, it would be most desirable to prevent the promoters



from interfering with one another. This could be achieved either by placing two promoters into the construct such that they direct transcription in opposite directions, or by inserting transcriptional terminator sequences downstream of each separately transcribed unit. The main advantage of the multiple-promoter construct is that it 5 permits independent regulation of as many distinct units as desired, which could be important. The disadvantages are that it would be more difficult to construct; more difficult to amend later; and more difficult to effectively regulate, since multiple changes in fermentation conditions would need to be introduced and might render the performance of the fermentation somewhat less predicable.

In any construct, the promoter sequence(s) used should be functional in the selected host organism and preferably provide sufficient transcription of the genes comprising the glycerol to 3 - hydroxypropionic acid pathway to enable the construct to be adequately active in that host. The promoter sequence(s) used would also effect regulation of transcription of the genes enabling the glycerol to 3-HP pathway to be 15 adequately active under the fermentation conditions employed for 3-HP production, and preferably they would be inducible, such that expression of the genes could be modulated by the inclusion in, or exclusion from, the fermentation of a certain agents or conditions.

A plausible example of the use of such a construct follows: one promoter, which 20 induced by the addition of an inexpensive chemical (the inducer) to the medium, could control transcription of both the dhaB gene and the gene encoding the appropriate aldehyde dehydrogenase. The cells would be permitted to grow in the absence of the inducer until they accumulated to a predetermined level. The inducer would then be added to the fermentation and nutritional changes commensurate with the altered 25 metabolism would be made to the medium as well. The cells would then be permitted to utilize the substrate(s) provided for 3-HP production (and additional biomass production if desired). After the cells could no longer use substrate to produce 3-HP, the fermentation would be stopped and the 3-HP recovered.

Genetic Sequences

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To express glycerol dehydratase and a suitable aldehyde dehydrogenase, the two

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enzymes necessary for the production of 3 - hydroxypropionic acid from glycerol, it is required that the DNA sequences containing the glycerol dehydratase and aldehyde dehydrogenase coding sequences be combined with at least a promoter sequence (preferably a non-native promoter although some native promoter activity may be present). An exemplary method of construction is described in the example below. To ensure that the present specification is enabling, the full sequences of the coding regions of genes for these enzymes is presented here.

Sequences 1, 3, 5 and 7 present different native genomic sequences for genes encoding aldehyde dehydrogenases.

SEQ ID NO:1 contains the full native DNA sequence encoding the *ALD4* enzyme from *Saccharomyces cerevisiae*. The amino acid sequence of the protein is presented as SEQ ID NO:2.

SEQ ID NO:3 includes the DNA sequence for the human ALDH2 gene, again including the full protein coding region. The amino acid sequence for this human alcohol dehydrogenase is presented in SEQ ID NO:4.

SEQ ID NO:5 and 7 respectively present the full coding sequences from the E. coli genes aldA and aldB, both of which encode alcohol dehydrogenases. The amino acid sequences for the proteins encoded by the genes are presented in SEQ ID NO: 6 and 8 respectively.

SEQ ID NO:9 contains the native genomic DNA sequence for the *dhaB* gene from the *dha* regulon of *Klebisiella pneumoniae*. The coding sequences for this complex regulon produces five polypeptides, which are presented as SEQ ID NOS:10 through 13, which together provide the activity of the glycerol dehydratase enzyme.

Each of these coding sequences can be used to make genetic constructs for the

25 expression of the appropriate enzymes in a heterologous hosts. In making genetic

constructs for expression of the genes in such hosts, it is contemplated that heterologous

promoters will be joined to the coding sequences for the enzymes, but all that it required

is that the promoters be effective for the hosts in which the genes are to be expressed. It

is also contemplated and envisioned that significant variations in DNA sequence are

30 possible from the native DNA coding sequences presented here. As is well known in

the art, due to the degeneracy of the genetic code, many different DNA sequences can

encode the expression of the same protein. So, when this document uses language specifying a DNA sequence encoding a protein, it is intended to encompass any DNA sequence which can be used to express that protein even if different from the genomic sequences presented here. It is also contemplated that conservative changes in the

5 amino acid sequences of the proteins specified here can be made without departing from the present invention. In particular, deletions, additions and substitutions of one or more amino acids in a protein sequence can almost always be made without changing protein functionality. When the name of a protein is sued here, it is intended to be equally applicable to both such minor changes in amino acid sequence and to allelic variations in native protein sequence as occurs within the species named as well as other closely related species.

It is possible that many of the above DNA sequences could be truncated and still express a protein that has the same enzymatic properties. One skilled in the art of molecular biology would appreciate that minor deletions, additions and mutations may not change the attributes of the designated base pair sequences; many of the nucleotide of the designated base pair sequences are probably not essential for their unique function. To determine whether or not an altered sequence or sequences has sufficient homology with the designated base pairs to function identically, one would simply create the candidate mutation, deletion or alteration and create a gene construct including the altered sequence together with promoter and termination sequences. This gene construct could be tested as, described below, for the production of 3-HP from glycerol.

Certain DNA primers were used to isolate or clone the genomic DNA sequences used in the experiments described below. While the sequence information presented

25 here is sufficient to enable the construction of expression plasmids incorporating the genes identified here, in order to redundantly enable the use of these genes, primers which may be used to isolated the genes from their native hosts are described below.

The primers aldA_L (SEQ ID NO:14), and aldA_R (SEQ ID NO:15), were used to amplify the 1513 bp aldA fragment from genomic E. coli DNA (strain MG1655, a gift from the Genetic Stock Center, New Haven, CT). The gel purified PCR fragment containing a DNA sequence coding for the expression of aldehyde dehydrogenase was

inserted into *NcoI-XhoI* site of pSE380 (Invitrogen, San Diego, CA) to give pPFS3. The resulting plasmid contained *aldA* under the control of the *trc* promoter. This construct allowed for high-level expression of the *aldA* gene from *E. coli* under regulation of the *trc* promoter. Unless indicated otherwise all molecular biology and plasmid constructions were done in *E. coli* AG1 (Stratagene, La Jolla, CA).

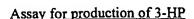
The primers aldB_L (SEQ ID NO:20) and aldB_R (SEQ ID NO:21), were used to amplify the 1574 bp aldB fragment from genomic E. coli DNA (strain MG1655). The resulting PCR converted the TGA stop codon into a TAA stop codon. The gelpurified PCR fragment containing the DNA sequence sufficiently coding for the expression of aldehyde dehydrogenase was inserted into the KpnI-SacI site of pSE380 to give pPFS12.

The primers ALD4_L (SEQ ID NO: 16), and ALD4_R (SEQ ID NO: 17), were used to amplify the 1595 bp ALD4 fragment from S. cerevisiae DNA (strain YPH500).

The gel-purified fragment containing a DNA sequence coding for the expression of aldehyde dehydrogenase was inserted into the KpnI-SacI site of pPFS3 to give pPFS8. The resulting plasmid contained mature ALD4 under control of the trc promoter.

The primers ALDH2_L (SEQ ID NO:18), and ALDH2_R (SEQ ID NO:19), were used to amplify the 1541 bp ALDH2 fragment from pT7-7::ALDH2, a gift from H. Weiner (Purdue University, West Lafayette, IN). The gel purified PCR fragment containing a DNA sequence sufficiently homologous to base pairs 22 to 1524, inclusive of SEQ ID NO: 3 so as to code for the expression of aldehyde dehydrogenase was inserted in to the *KpnI-SacI* site of pSE380 to give pPFS7. This sequence was moved from pPFS7 into the *KpnI-SacI* site of pPFS3 to give pPFS9. The resulting plasmid contained mature ALDH2 under the control of the *trc* promoter.

The primers pTRC_L (SEQ ID NO:22), and pTRC_R)SEQ ID NO:23), were used to amplify the 540 bp fragment from pSE380. The gel purified PCR fragment was inserted into the *Hpal-Kpnl* site of pPFS3 to give pPFS13. The resulting plasmid deleted the "native" ribosome binding site of pSE380 and a *Ncol* site (which contained an extraneous ATG start codon upstream of the cloned genes). The *Kpnl-Sacl* fragments of pPFS8, pPFS9, and pPSF12 were inserted into the *Kpnl-Sacl* site of pPFS13 to give pPFS14, pPFS15, and pPFS16, respectively.



The efficacy of changes made as contemplated herein can be checked by the following tests. To test for the production of 3-HP, fermentation products can be quantified with a Waters Alliance Integrity HPLC system (Milford, MA) equipped with a refractive index detector, a photodiode array detector, and an Aminex HPX-87H (Bio-Rad, Hercules, CA) organic acids column. The mobile phase should be 0.01 N sulfuric acid solution (pH 2.0) at a flow rate of 0.5 mL/min. The column temperature should be set to 40°C. Compounds can be identified by determining if they co-elute with authentic standards. Prior to analysis, all samples should be filtered through 0.45 µM pore size membrane. (Gelman Sciences, Ann Arbor, MI). The fractions of the fermentation products collected using HPLC should be analyzed on a Varian Star 3400 CX, gas - chromatograph coupled to a Varian Saturn 3 mass spectrometer (GC-MS) (Walnut Creek, CA).

Assay for enzyme activity.

Aldehyde dehydrogenase activity can be determined by measuring the reduction of β-NAD⁺ at 25°C with 3 - hydroxypropionaldehyde as a substrate. All buffers should contain 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM Pefabloc SC (Boehringer Mannheim, Indianapolis, IN) and 1 mM Tris (carboxyethyl) phosphine hydrochloride (TCEP-HCL). For ALD4, the solution should contain 100 mM Tris HCL Buffer (pH 8.0), 100 mM KCl. For ALDH2 the solution should contained 100 mM sodium pyrophosphate (pH 9.0). For AldA and AldB, the solution should contain 20 mM sodium glycine (pH 9.5). A total of 3.0 mL of buffer should be added to quartz cuvettes and allowed to equilibrate to assay temperature. From 5 to 20 μL of cell extract should be added and background activity recorded after the addition of β-NAD⁺ to a final concentration of 0.67 mM. The reaction should be started by the addition of substrate (either acetaldehyde, propionaldehyde, or 3 - hydroxypropionaldehyde) to a final concentration of 2 mM. Assay mixtures should be stirred with micro-stirrers during the assays.

For aldehyde dehydrogenase activity assays, one unit is defined as the reduction

of 1.0 μM of β-AND⁺ per minute at 25° C. These reactions can be monitored by following the change in absorbence at 340 nm (A₃₄₀) at 25°C on a Varain Carry-1 Bio spectrophotometer (Sugar Land, TX). Total protein concentrations in the cell extracts can be determined using the Bradford assay method (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

EXAMPLES

Plasmid constructions.

Klebsiella pneumoniae expresses glycerol dehydratase, an enzyme that catalyzes the conversion of glycerol to 3 - hydroxypropionaldehyde, (dhaB) and 1,3 propanediol oxidoreductase an enzyme that catalyzes the conversion of 3 - hydroxypropionaldehyde to 1,3 - propanediol respectively (the gene product from dhaT). A plasmid encoding these two genes was created and expressed in E. coli (plasmid pTC53). The dhaT gene was deleted from pTC53 to create pMH34. The resulting plasmid still contained the DNA sequence complementary to base pairs 330 to
2153 inclusion of SEQ ID NO: 9, the complement of base pairs 2166 to 2591, inclusive, of SEQ ID NO: 9, and the complement of base pairs 3191 to 4858, inclusive, of SEQ ID NO: 9, so as to code for the expression of glycerol dehydratase. The fragment of DNA encoding these sequences was excised from pMH34 by cutting it with Sal1-Xbal, and the resulting fragment was gel purified (the purified fragment was gift
from M. Hoffman of the University of Wisconsin - Madison). This DNA fragment was inserted into the Sal1-Xbal site of pPFS13 to give pPFS17.

The resulting plasmid contained both the aldA and dhaB genes under the control of the trc promoter. Similarity, the gel-purified Sall-Xbal fragment from pMH34 was inserted into the Sall-Xbal sites of pPFS14, pPFS15, and pPFS16 to give pPFS18, pPFS19, and pPFS20, respectively. These plasmids contained ALD4, ALDH2, and aldB, respectively, as well as dhaB under the control of the trc promoter; in all of the constructs the dhaB gene were downstream of the gene encoding the aldehyde dehydrogenase.



Expression in E. coli.

The efficacy of *E. coli* as a platform for the production of 3-HP from growth on glucose has been examined using a mathematical model developed for this purpose. The model was executed in two different ways assuming the conversion of one mole of glucose under either anaerobic or aerobic conditions either directly to 3-HP or to the production of 3-HP and ATP. The optimum yield under anaerobic conditions is 1 mole of 3-HP and 1 mole of lactate. The more realistic yield under anaerobic conditions is 0.5 moles of 3-HP, 1.5 moles of lactate and 1 mole of ATP. The optimum yield under aerobic conditions is 1.9 moles of 3-HP and 0.3 moles of CO₂. The realistic yield under aerobic conditions is 1.85 moles of 3-HP, 0.35 moles of CO₂ and 1 mole of ATP.

The effect of 3-HP concentration on *E. coli* strain MG1655 growth was measured. Cells were grown on standard media with and without the addition of up to 80g/L of 3-HP. The best fit of these data demonstrated that 3-HP was only 1.4 times as inhibitory as lactic acid on the growth of *E. coli*. It is possible to economically produce lactic acid using *E. coli*, since 3-HP is only 1.4 times more inhibitory than lactic acid, it should be possible to use *E. coli* as a host for the commercial production of 3-HP.

Media and growth conditions

The standard media contained the following per liter: 6 g Na₂HPO₄, 3 g KH₂PO₄, 1 g NH₄Cl, 0.5 g NaCl, 3 mg CaCl₂, 5 g yeast extract (Difco Laboratories, Detroit, MI) and 2 mM MgSO₄. When necessary to retain plasmids ampicillin (100 mg/mL) was added to the media. Isopropyl-β-thiogalactopyranoside (IPTG) was added in varying amounts to induce gene expression. All fermentations were carried out in an incubator-shaker at 37 C and 200 rpm. Anaerobic fermentations were carried out in 500-mL anaerobic flasks with 300 mL of working volume. Inocula for fermentations were grown overnight in Luria-Bertani medium supplemented with ampicillin is necessary. The 300-mL fermentations were inoculated with 1.5 mL of the overnight culture. For enzyme assays, fermentations were incubated for 24 hours.

Over expression of aldehyde dehydrogenase in E. coli.

Cells were harvested by centrifugation at 3000 x g for 10 minutes at 4°C with a



Beckman (Fullerton, CA) model J2-21 centrifuge. Cell pellets were washed twice in 100 mM potassium phosphate buffer at pH 7.2 and re-suspended in appropriate assay resuspension buffer equal to 5 x of the volume of the wet cell mass. The cells were homogenized using a French pressure cell. The homogenate was centrifuged at 40000 x g for 30 minutes. The supernatant was dialyzed against the appropriate resuspension buffer using 10000 molecular weight cut-off pleated dialysis tubing (Pierce, Rockford, IL) at 4°C. Dialysis buffer was changed after 2 hours, and 4 hours, and dialysis was stopped after being allowed to proceed overnight.

E. coli AG1 cells transfected with the plasmids constructed to express the aldA,
10 ALD4, ALDH2, or aldB genes were grown in 500-mL anaerobic flasks. Twelve hours after the fermentations were inoculated IPTG was added to induce enzyme expression. The cells were allowed to grow for an additional 12 hours then harvested and lysed as discussed above. The soluble fraction of the lysate was assayed for aldehyde dehydrogenase activity using the substrate 3-hydroxypropionicaldehyde in the buffer
15 appropriate for the particular enzyme expressed The plasmid, aldehyde dehydrogenase expressed and specific activity measured (U/mg of protein) were as follows: pPFS13, aldA, 0.2; pPFS14, ALD4, 0.5, pPFS15, ALDH2, 0.3; and pPFS16, aldB. 0.1. The control, E. coli strain AG1 harboring plasmid pSE380, encoded no exogenous aldehyde dehydrogenase activity and it had no detectable activity with 3-HP as substrate. It is
20 clear from the activity assays that all four aldehyde dehydrogenases were expressed in E. coli. The aldehyde dehydrogenase cloned from Saccharomyces cerevisiae (ADH4) had the highest activity when 3-hydroxypropionaldehyde was used as the substrate (0.5 units/mg of protein).

E. coli cells transformed with plasmids expressing: aldehyde dehydrogenase;
both aldehyde dehydrogenase and glycerol dehydratase, or neither gene; were grown and assayed for their ability to produce 3-HP from glycerol. The cells were grown on standard media supplemented with 6 μM of Coenzyme B₁₂, under anaerobic conditions in the absence of light (to protect the integrity of the Coenzyme B₁₂ necessary for DhaB activity). After 12 hours, IPTG was added to induce expression of the genes under the
trc promoter at the same time 5g/L of glycerol was added. After 12 more hours of anaerobic fermentation the fermentation broth was assayed for 3 - HP by HPLC and GC,

the plasmid, aldehyde dehydrogenase gene expressed and g/L of 3- HP measured were as follows: pSF17, aldA, 0.031; pPSF18 ALD4, 0.173; and pPSF19, ALDH2, 0.061. Cells expressing dhaB but no exogenous aldehyde dehydrogenase genes (plasmid pMH34) produced 0.015 g/L of 3 - HP. Cells expressing aldA, ALD4, ALDH2 or aldB 5 but not dhaB (plasmids pPFS13, pPFS14, pPFS15, pPFS16, respectively) all produced less then 0.005 g/L of 3-HP when the media the cells were growing in was supplemented with 2.5g/L of 3-hydroxypropionaldehyde.

Other Hosts and Promoters

Applications of the 3 - hydroxypropionic acid pathway such as the genetic 10 constructs of the present invention can easily be expressed in other organisms. The required genes would need to be placed under control of an appropriate promoter or promoters. Some organism such as yeasts may require transcription terminators to be placed after each transcribed unit. The knowledge of the present intention makes such amendments possible. Such a genetic construct would need to be part of a vector that 15 could either replicate in the new host or integrate into the chromosome of the new host. Many such vectors are commercially available for expression in gram-negative and gram-positive bacteria, yeast, mammalian cells, insect cell, plant, etc. For example, to express the 3-hydroxypropionic acid pathway in Rhodobacter capsulatus, one could obtain vector pNH2 from the American Type Culture Collection (ATTC). This is a 20 shuttle vector for use in R. capsulatus and E. coli. Organisms such as Saccharomyces cerevisiae which can convert glucose to glycerol could be used as a host, such a construct would enable the production of 3 - HP directly from glucose. Additionally, other substrates such as xylan could also be used given the selection of an appropriate host.

Stochiometric analysis shows that best stochiometric yield of 3-HP production in E. coli calculated on the basis of glucose consumed is obtained under aerobic conditions. Under aerobic condition CO₂ is the only carbon-containing co-product, in particular the generation of lactic acid which occurs under anaerobic conditions is avoided. Production of 3-HP under these conditions could result in a more economical 30 recovery of 3-HP from the fermentation broth.

Alternatively, the *dhaB* gene and a gene encoding the appropriate aldehyde dehydrogenase could be cloned into the multiple cloning site of this vector in *E. coli* to facilitate construction, and then transformed into *R. capsulatus*. The *R. capsulatus* nifH promoter, provided on the plasmid, could be used to direct the transcription in *R. capsulatus* of the genes placed into pNF2 in series with one promoter, or with two copies of the nifH promoter. Expression of the genes in other organisms would require a procedure analogous to that presented here.

Alternative Aldehyde Dehydrogenases and Glycerol Dehydratases

Applications of the pathway for the production of 3-hydroxypropionic acid from glycerol can be made using other suitable aldehyde dehydrogenases. To be functional in this pathway an aldehyde dehydrogenase needs to be stable, readily expressed in the host of choice and have high enough activity towards 3-hydroxypropionaldehyde to enable it to make 3-HP. The knowledge of the present invention makes such amendments possible. A program of directed evolution could be undertaken to select for suitable aldehyde dehydrogenases or they could be recovered from native sources, the genes encoding these enzymes in conjunction with a gene encoding an appropriate glycerol dehydratase activity, would then be made part of any of the constructs envisioned here to produce 3 - hydroxypropionic acid from glycerol.

A similar program of enzyme improvement including for example directed

evolution could be carried out using the *dhaB* gene from *Klebsiella pneumoniae* as a

starting point to obtain other variants of glycerol dehydratase that are superior in

efficiency and stability to the form used in this invention. Alternatively, enzymes which

catalyzes the same reaction may be isolated from others organisms and used in place of

the *Klebsiella pneumoniae* glycerol dehydratase. Such enzymes may be especially

useful in alternative hosts wherein they may be more readily expressed, be more stable

and more efficient under the fermentation conditions best suited to the growth of the

construct and the production and recovery of 3-HP.

CLAIM OR CLAIMS

I/WE CLAIM:

- 1. A method for producing 3-hydroxypropionic acid comprising the steps of providing in a fermenter a recombinant microorganism which expresses genes
- 5 for non-native enzymes which are capable of catalyzing the production of 3hydroxypropionic acid from glycerol;

providing a source of glycerol or glucose for the recombinant microorganism, and

fermenting the microorganism under conditions which result in the accumulation of 3-hydroxypropionic acid.

A method for producing 3-hydroxypropionic acid comprising the steps of providing in a fermenter a recombinant microorganism which carries genetic constructions for the expression of a glycerol dehydratase and an aldehyde dehydrogenase which are capable of catalyzing the production of 3-hydroxypropionic
 acid from glycerol;

providing a source of glycerol or glucose for the recombinant microorganism, and

fermenting the microorganism under conditions which result in the accumulation of 3-hydroxypropionic acid.

- 3. A method for producing 3-hydroxypropionic acid comprising the steps of providing in a fermenter a recombinant microorganism which carries a genetic construct which expresses the *dhaB* gene from *Klebsiella pneumoniae* and a gene for an aldehyde dehydrogenase, which are capable of catalyzing the production of 3-
- 5 hydroxypropionic acid from glycerol;

providing a source of glycerol or glucose for the recombinant microorganism, and

fermenting the microorganism under conditions which result in the accumulation of 3-hydroxypropionic acid.

- 4. The method of claim 3 wherein the gene for the aldehyde dehydrogenase is selected from the group consisting of ALDH4, ALD2, aldA and aldB.
 - 5. The method of claim 3 wherein the aldehyde dehydrogenase is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:8.
- 6. A recombinant E. coli host comprising in its inheritable genetic materials
 15 foreign genes encoding a glycerol dehydratase and an aldehyde dehydrogenase, such that the host is capable of producing 3-hydroxypropionic acid from glycerol.
 - 7. A recombinant *E. coli* host comprising in its inheritable genetic materials the *dhaB* gene from *Klebsiella pheumoniae* and the *ald4* gene from *Saccharomycetes* cervisiae, such that the host is capable of producing 3-hydroxypropionic from glycerol.

- 8. A bacterial host comprising in its inheritable genetic material a genetic construction encoding for the expression of a glycerol dehydratase enzyme and an aldehyde dehydrogenase enzyme, such that the bacterial host is capable of converting glycerol to 3-hydroxypropionic acid.
- 5 9. The bacterial host of claim 8 wherein the glycerol dehydratase from *Klebsiella pneumoniae*.
 - 10. The bacterial host of claim 8 wherein the gene encoding the glycerol dehydratase is the *dhaB* gene from *Klebsiella pneumoniae*.
- 11. The bacterial host of claim 8 wherein the aldehyde dehydrogenase is
 selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:8.
 - 12. The bacterial host of claim 8 wherein the gene for the aldehyde dehydrogenase is selected from the group consisting of ALDH4, ALD2, aldA and aldB.



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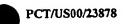
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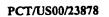
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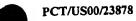
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155

165



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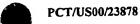
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245



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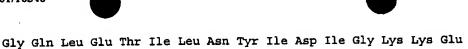


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365



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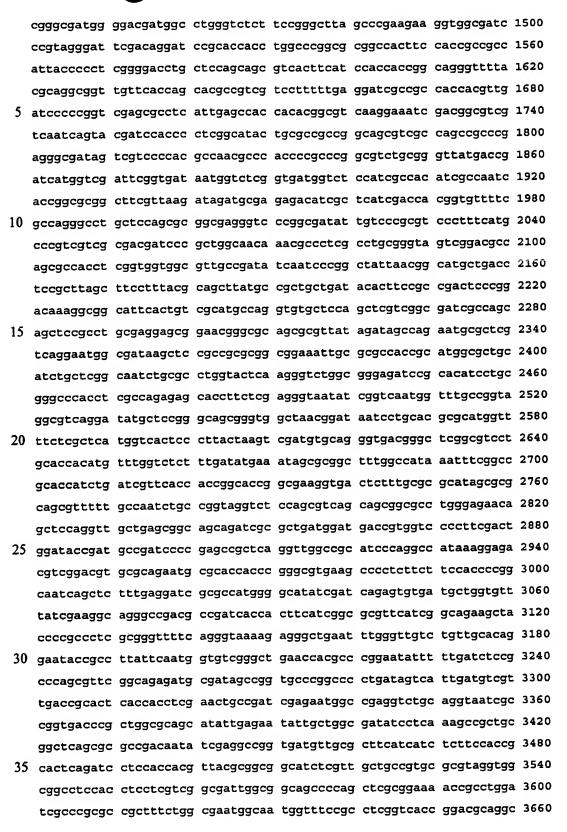
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- Asp Val Ala Met Glu Thr Ile Thr Glu Thr Ile Ile Thr Glu Ser Thr 10 85 90 95
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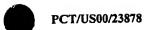


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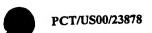
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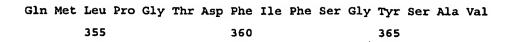
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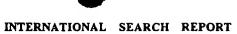
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International application No. PCT/US00/23878

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	:C12P 7/22; C12N 9/02, 9/14, 1/20, 15/00		
	:435/155, 189, 195, 252.3, 320.1 to International Patent Classification (IPC) or to both	h national classification and IPC	
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	documentation searched (classification system follows	ed by classification symbols)	<u> </u>
	435/155, 189, 195, 252.3, 320.1	or of committee of the original of the origina	
Documenta	tion searched other than minimum documentation to th	e extent that such documents are included	in the fields searched
	data base consulted during the international search (n		e, search terms used)
C. DOC	UMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where a	ppropriate, of the relevant passages	Relevant to claim No.
A	TANG et al. Immunochemical Pro- Glycerol Dehydrogenases from Esch pneumoniae. Journal of Bacteriology. No. 3, pages 1169-1174, see the entire	nerichia coli and Klebsiella December 1982, Vol. 152,	1-12
A	BARBIRATO et al. Anaerobic pathway Enterobacter agglomerans CNCM 1210 Microbiology. 1997, Vol. 143, No. 7	): limitations and regulations.	1-12
Furt	ner documents are listed in the continuation of Box C	C. See patent family annex.	
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Date of the	actual completion of the international search	Date of mailing of the international se	arch report
16 OCTO	BER 2000	VON 0 R	2000
Commission Box PCT	nailing address of the ISA/US ner of Patents and Trademarks a, D.C. 20231	Authorized officer TEKCHAND SAIDHA	3 vides
Facsimile N		Telephone No. (703) 308-0196	9

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